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A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo

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We describe a protein with the hallmarks of a chemokine, designated CXCL16, that is made by dendritic cells (DCs) in lymphoid organ T cell zones and by cells in the splenic red pulp. CXCL16 contains a transmembrane domain and both membrane-bound and soluble forms are produced. Naïve CD8 T cells, natural killer T cells and a subset of memory CD4 T cells bind CXCL16, and activated T cells migrated chemotactically to the soluble chemokine. By expression cloning, Bonzo (also known as STRL33 and TYMSTR) was identified as a CXCL16 receptor. CXCL16 may function in promoting interactions between DCs and CD8 T cells and in guiding T cell movements in the splenic red pulp. CXCL16 was also found in the thymic medulla and in some nonlymphoid tissues, indicating roles in thymocyte development and effector T cell trafficking.

Effective induction of adaptive immunity requires rapid encounter between antigen presenting cells and rare antigen-specific lymphocytes in peripheral lymphoid organs. Therefore, the effector phase of cell-mediated immune responses depends on efficient dispersal of activated antigen-specific cells. Molecules of the chemotactic cytokine, or chemokine, family have long been known to help recruit activated T cells to sites of inflammation[1]. Chemokines are also involved in regulating movements of lymphocytes and antigen presenting cells in lymphoid organs[2].

Chemokines form a large family of small, structurally related proteins[3,4]. Four subfamilies have been defined, based on the number and spacing of cysteine residues in the amino-terminal region, as C, CC, CXC and CX3C chemokines. All are secreted proteins except the single CX3C chemokine, CX3CL1 (fractalkine), in which the chemokine domain is followed by a mucin-type stalk, a transmembrane domain and a cytoplasmic tail[5,6]. Chemokines signal by binding receptors that are members of the seven-transmembrane G protein–coupled family[7]. Several members of this family, in particular CXCR4 and CCR5, also function as coreceptors for the human immunodeficiency virus (HIV)[8]. Reciprocally, several HIV coreceptors have been isolated that so far lack known chemokine ligands and are therefore termed orphan chemokine receptors[8].

Although an understanding has begun to emerge of the chemokines that regulate cell movements in the lymphoid regions of spleen and lymph nodes, less is understood about the molecules directing cells through other parts of these tissues. In particular, the red pulp of spleen is an important site of trafficking for many leukocyte types[9,10]. Activated and effector CD8 cells become concentrated in the splenic red pulp during viral infections[11]. Natural killer (NK) cells in the spleen predominantly localize in the red pulp[12] and short-lived plasma cells migrate into this compartment to secrete antibody[13]. The factors controlling these positional events remain obscure.

We now characterize a transmembrane CXC chemokine, CXCL16, that is made by T cell zone DCs and by cells in

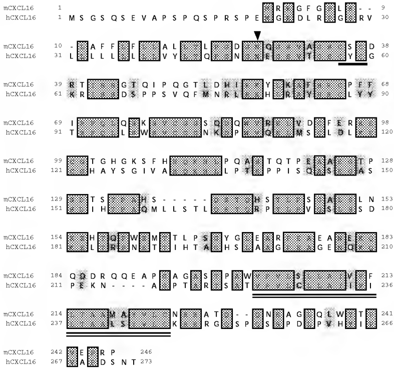

Figure 1. Amino acid sequence and alignment of mouse and human CXCL16. The CXC motif is underlined and the transmembrane domain is double-underlined. Arrowhead designates the first amino acid of the mature mouse CXCL16, as determined by amino-terminal sequencing of CXCL16 made in J558L cells. Alignment was generated by ClustalW program. Identical residues appear in bold type and are boxed; similar amino acids are shaded lightly. Nucleotide sequences have been deposited at GenBank and accession numbers are AF301016 (hCXCL16) and AF301017 (mCXCL16).

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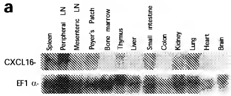
the splenic red pulp and is up-regulated after exposure to inflammatory stimuli. CXCL16 receptors are expressed on naïve CD8 cells and in larger amounts on intraepithelial lymphocytes (IELs), natural killer T (NKT) cells and activated CD8 and CD4 T cells. Using an CXCL16-Fc fusion protein in expression cloning, we identified the HIV coreceptor Bonzo (also called STRL33 and TYMSTR)[14–16] as a CXCL16 receptor. Thus, CXCL16 may function in T cell–DC interactions and in regulating movements of activated T cells in the splenic red pulp and in peripheral tissues.



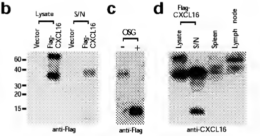


Figure 2. Northern and western blot analyses of murine CXCL16. (**a**) Northern blot showing levels of CXCL16 mRNA in the indicated mouse tissues. EF1-α hybridization indicates amount of total RNA loaded in each lane. (**b–d**) Detection of cell-associated and soluble CXCL16 by western blot. Flag-tagged proteins from lysates or supernatants of HEK293 cells transfected with N-terminal Flag-tagged CXCL16 were immunoprecipitated with anti-Flag, separated on SDS-PAGE and detected by blotting with anti-Flag (**b, c**) or rabbit anti-CXCL16 (**d**). In (**c**) Soluble Flag-tagged proteins were immunoprecipitated from supernatants of Flag-CXCL16–expressing cells that were either untreated or treated with O-sialoglycoprotein endopeptidase (OSG). In (**d**) Lanes 3 and 4 contain protein prepared from mouse spleen and lymph node lysates. The position of molecular weight standards (kD) are shown. (LN, lymph node; S/N, supernatant.)

Results

Identification of a transmembrane CXC chemokine

To identify new proteins of immunological interest, we conducted a two-step search of public domain human expressed sequence tag (EST) databases. First, overlapping ESTs were identified and assembled into contiguous coding sequences with each representing a putative transcript. Then, PROSITE[17] was used to scan the protein encoded by the assembled transcript for occurrence of functional patterns. This strategy led to identification of a sequence that resembled the chemokine family. Analysis of the full-length cDNA showed a non-ELR (glutamate-leucine-arginine) motif-containing CXC chemokine domain, a spacer region, a transmembrane domain and a cytoplasmic tail (**Fig. 1**). As this sequence represents the sixteenth identified CXC chemokine, we have named it CXCL16, in accordance with the consensus proposal on chemokine nomenclature[4]. Several homologous mouse EST clones were identified that correspond to a single cDNA with 49% overall amino acid identity and 70% similarity in the chemokine domain to human CXCL16. This similarity and the equivalent domain organization led us to conclude that we had identified murine CXCL16 (**Fig. 1**). CXCL16 represents the first transmembrane CXC chemokine and, with CX3CL1, the second transmembrane member of the chemokine family. The ~110aa spacer domain of CXCL16 is rich in serine, threonine and proline, as is typical of mucin structures[18]. A similar domain exists in CX3CL1[5,6]. Following the transmembrane domain, both mouse and human CXCL16 contained a small (24–27aa) cytoplasmic domain, with a YXPV motif that is a potential tyrosine-phosphorylation and SH2-protein–binding site. A similar motif, YXPR, is found in CX3CL1[5,6]. Human and murine CXCL16 are also unusual in that they contain six cysteines in the chemokine domain, a property previously only observed in a subfamily of CC chemokines[3]. By analyzing a panel of human–hamster somatic-cell hybrids, human CXCL16 was mapped to chromosome 17p13. This places CXCL16 on a separate locus to all other known chemokines[3].

Organ distribution of CXCL16 mRNA

Northern blot analysis for mouse CXCL16 showed a predominant 2.2-kb band in RNA from spleen, lymph nodes and Peyer's patches (**Fig. 2a**). In primary lymphoid organs, expression was detected in thymus but not bone marrow (**Fig. 2a**). Expression was also notable in several nonlymphoid tissues including lung, small intestine and kidney, and weak expression was observed in liver and heart (**Fig. 2a**). Little or no expression could be detected in brain (**Fig. 2a**) or in purified T or B lymphocytes (data not shown). A similar pattern of human CXCL16 mRNA expression was seen in normal human tissues (data not shown). Therefore, CXCL16 seems likely to function in regulating the trafficking or interactions of cells in peripheral lymphoid tissues, thymus and in some nonlymphoid organs.

Characterization of CXCL16 protein species

To determine whether CXCL16 exists solely as a transmembrane protein or whether it can also occur in a soluble form, we expressed the murine protein with an amino-terminal Flag tag in HEK293 cells and tested for Flag-tagged proteins in cell lysates and culture supernatants. Cell surface expression of Flag-CXCL16 was confirmed by flow cytometry (data not shown). Cell lysates contained two principal Flag-tagged proteins of ~60 and ~34 kD, whereas the culture supernatants contained a ~35-kD form and small amounts of a ~12-kD form (**Fig. 2b**). No anti-Flag–reactive material was present in lysates or supernatant from cells transfected with the empty vector (**Fig. 2b**). We speculate that the ~60-kD cell-associated form corresponds to the full length transmembrane chemokine and that the higher apparent molecular weight relative to the mass of ~24 kD predicted by the primary sequence is due to heavy glycosylation of the

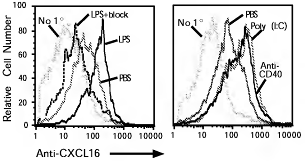


Figure 3. Cell surface expression of CXCL16 on DCs. Cell preparations from spleens of mice treated with PBS, LPS, poly(I)•poly(C) or stimulating anti-CD40 were prepared with mild collagenase treatment and analyzed for expression of CXCL16 on $CD11c^+$ DCs using rabbit anti-CXCL16. Background staining in the absence of anti-CXCL16 is shown as a control (No. 1°). Pre-absorption of the polyclonal antiserum to CXCL16 (LPS+block) reduced the amount of fluorescent signal to that of the background, showing the specificity of DC staining with this reagent.

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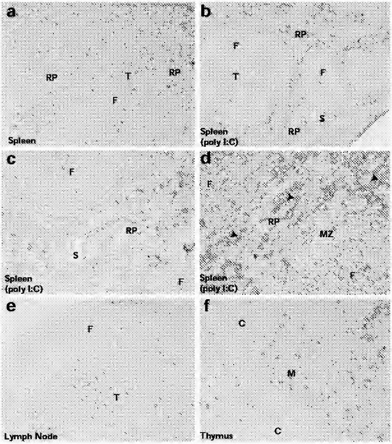

Figure 4. Expression of CXCL16 in spleen, lymph node and thymus. Naïve mouse spleen (**a**); poly(I)•poly(C) treated mouse spleen (**b–d**); lymph node (**e**); and thymus (**f**) were stained blue with rabbit anti-CXCL16. (**c**) Higher magnification of CXCL16 expressing linear structures leading into a sinus. In (**d**) CD8 cells are stained brown and arrowheads indicate examples of the close proximity of these cells to CXCL16-expressing structures. Original magnifications are ×5 (**a, b**), ×20 (**c, d**) and ×10 (**e, f**). (T, T zone; F, follicle; MZ, marginal zone; RP, red pulp; S, sinus; C, cortex; M, medulla.)

mucin-like spacer domain. Consistent with this, treatment of HEK293 cells expressing Flag-CXCL16 with O-sialoglycoprotein endopeptidase (OSG), a protease that specifically cleaves O-glycosylated mucin domains[19], resulted in diminished cell surface expression of Flag-CXCL16 as determined by flow cytometry (data not shown) and release of a soluble ~12-kD fragment (**Fig. 2c**). Supernatants of cells that were not treated with OSG (**Fig. 2c**) contained small amounts of the ~35-kD soluble form, which most likely corresponds to a fragment containing the ~10-kD chemokine domain and part of the mucin stalk, released from the membrane by endogenous metalloproteases. Several sequences resembling metalloprotease recognition sites[20] exist in the stalk region. At present, it is unclear whether the ~34-kD species detected in cell lysates (**Fig. 2b**) corresponds to a proteolytically cleaved form or an intracellular precursor.

The same soluble and cell-associated proteins are recognized by an affinity-purified rabbit antiserum specific for the murine CXCL16 (mCXCL16) chemokine domain (**Fig. 2d**). Using the antiserum, we identified two predominant groups of bands in total protein extracts of spleen and lymph nodes that correspond to the ~60-kD and the ~35-kD bands detected in the transfected cells (**Fig. 2d**). The basis for the multiple bands is not known but may reflect differences in the number or types of O-linked sugars attached to the spacer domain. The small ~12-kD band seen in supernatants of Flag-CXCL16–transfected HEK293 cells was not detected in lymphoid organ lysates (**Fig. 2d**). In summary, these observations indicate that mCXCL16 is present in lymphoid tissues in both membrane-bound and soluble forms. The proportions of these forms varied between spleen and lymph node preparations from different animals but, on average, they were present in similar amounts.

Membrane CXCL16 expression on dendritic cells

To identify cell types in peripheral lymphoid tissues expressing CXCL16, we used the affinity-purified antiserum in flow cytometric analysis. Expression of CXCL16 was found on CD11c$^+$ splenic and lymph node DCs (**Fig. 3**), whereas no expression was detected on T or B cells. Analysis of DC subsets showed higher constitutive expression on CD8$^+$CD11c$^+$ cells compared with CD8$^-$CD11c$^+$ cells (data not shown). Intraperitoneal injection of mice with lipopolysaccharide (LPS), a potent activator of DCs, led to a fivefold increase in expression of CXCL16 on spleen and lymph node DCs (**Fig. 3**). Similar CXCL16 up-regulation on DCs was seen following treatment of mice with a stimulating antibody to CD40 and with the synthetic double-stranded RNA, polyriboinosinic polyribocytidlyic acid poly(I)•poly(C); **Fig. 3**). Thus, the baseline expression of CXCL16 on DCs is up-regulated by inflammatory mediators that condition DCs to become potent antigen presenting cells.

CXCL16-expressing cell distribution in lymphoid tissues

Consistent with the detection of CXCL16 on DCs by flow cytometry, immunohistochemical analysis (**Fig. 4**) showed CXCL16 expression in T cell areas of the splenic white pulp (**Fig. 4a**) and lymph nodes (**Fig. 4e**). In the thymus, CXCL16-expressing cells were restricted to the medulla (**Fig. 4f**). There was no specific staining in the B cell areas of either the spleen or lymph node. In contrast, staining of similar intensity to the T zone staining was detected in the splenic red pulp (**Fig. 4a**). Because flow cytometric analysis has shown increased CXCL16 expression on DCs from animals exposed to inflammatory mediators, we tested whether these mediators increased the amount of CXCL16 that could be detected *in situ*. One day after injection of poly(I)•poly(C), CXCL16 expression was increased in T zones and, more prominently, in the splenic red pulp (**Fig. 4b**). A similar up-regulation occurred in both sites after *in vivo* exposure to LPS or anti-CD40 (not shown). Most of the CXCL16-expressing red pulp cells formed distinct elongated linear structures that led to red pulp sinuses, thus resembling small vascular channels (**Fig. 4b–d**). Little or none of the red pulp staining seemed to be associated with cells of dendritic morphology or with macrophages. After LPS injection, CXCL16 expression was sometimes detected on cells lining the large vascular sinuses (not shown). Because the principal function of DCs is to interact with T cells, it seems likely that CXCL16 is involved in DC–T cell interactions in the T zone. To examine whether the cells expressing CXCL16 in the red pulp also interact with T cells, sections were costained for CXCL16 and CD8. Close associations between CD8 cells and CXCL16-positive cells were readily observable (**Fig. 4d**).

Subpopulations that express CXCL16 receptors

To identify cell types able to bind CXCL16, we constructed an Fc fusion protein containing residues 1–122 of mCXCL16 and the hinge, CH2 and CH3 domains of human IgG1. Flow cytometric analysis of

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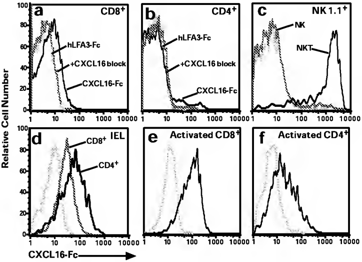

Figure 5. Expression of CXCL16 receptor by naïve and activated T cells. Various cell types were stained with CXCL16-Fc fusion protein. Intensity of staining is compared to staining with isotype control reagent, hLFA3-Fc (broken lines). Preincubation of cells with recombinant CXCL16 (CXCL16 block, solid lines in **a**, **b**) diminished the amount of CXCL16-Fc staining to that of the isotype control. NK and NKT cells were defined as NK1.1$^+$, CD3$^-$ and NK1.1^{lo}, CD3$^+$, respectively. For detection of Fc fusion on NK1.1$^+$ cells, a different antibody to human Fc was used that gives relatively brighter staining than the secondary reagent used for cell populations shown in the other panels.

spleen cells from naïve mice showed low CXCL16-Fc staining on all CD8 T cells (**Fig. 5a**), on a small (2–4%) subpopulation of CD4 T cells (**Fig. 5b**), but not on B cells, macrophages or neutrophils (data not shown). In the thymus, CXCL16-Fc showed weak staining of CD8$^+$ single-positive cells and 15–20% of the CD4$^-$CD8$^-$ double-negative population. Further characterization of peripheral CD4$^+$ cells identified the CXCL16-Fc$^+$ subpopulation as L-selectin^{lo} CD44^{hi} activated/memory phenotype. CXCL16-Fc binding to NK1.1^{lo} CD3$^+$ NKT cells was also detected (**Fig. 5c**). In contrast to the splenic T cells, there was high CXCL16-Fc staining of both CD4$^+$ and CD8$^+$ IEL (**Fig. 5d**). *In vitro* activation of splenic T cells with anti-CD3 and anti-CD28 led to a tenfold increase in CXCL16-Fc staining of CD8 cells (**Fig. 5e**) and a definite, but less notable, increase in staining of CD4 cells (**Fig. 5f**).

Chemotaxis of activated T cells to CXCL16

Using recombinant molecules containing the chemokine domain of CXCL16 in chemotaxis assays, we did not observe any chemotaxis of naïve CD8 T cells to CXCL16 (**Fig. 6a**), despite their expressing detectable amounts of receptors (**Fig. 5a**). Similarly, we did not detect any chemotaxis of naïve CD4 T cells, B cells, macrophages or neutrophils (**Fig. 6a** and data not shown). In contrast, CXCL16 induced a strong chemotactic response in activated CD8 cells, with migration of more than 40% of input cells (**Fig. 6b**). A lower proportion of *in vitro* activated CD4 cells migrated to CXCL16 (**Fig. 6b**), consistent with the lower amounts of the receptor on this population compared with activated CD8 cells (**Fig. 5e,f**). The migratory response of activated T cells was chemotactic rather than chemokinetic, as cells incubated in the absence of a chemokine gradient did not migrate (**Fig. 6c**). In addition, the chemotactic response was sensitive to pertussis toxin (**Fig. 6c**) and CXCL16 was effective at inducing calcium mobilization (**Fig. 6d**). These findings indicated that activated T cells express a functional CXCL16 receptor that is most likely a member of the seven-transmembrane $G\alpha_i$-coupled chemokine receptor family.

Identification of Bonzo as a CXCL16 receptor

We used an expression cloning approach to identify a receptor for CXCL16. A cDNA expression library made with mRNA from lymphokine-activated splenic CD8 T cells and NK cells was transfected into HEK293 cells and CXCL16-Fc^{high} cells were selected by FACS. After several cycles of selection and enrichment, we identified individual cDNA clones that, when expressed in HEK293 cells, showed strong specific binding to CXCL16-Fc (**Fig. 7a**). Complete sequencing of several of the clones showed a mouse protein with 71% amino acid sequence identity to the human orphan chemokine receptor and HIV/SIV coreceptor, Bonzo[14–16]. Therefore, Bonzo functions as a receptor for CXCL16 and, in accord with the consensus on chemokine receptor nomenclature[4], we renamed Bonzo as CXC chemokine receptor 6 (CXCR6). Stimulation of CXCR6-transfected HEK293 cells with CXCL16 resulted in a dose-dependent increase in intracellular calcium concentration (**Fig. 7b**). This calcium flux was not seen when cells transfected with either the empty vector or with another chemokine receptor, CCR7, were stimulated with CXCL16 (**Fig. 7b**). Stimulation of murine CXCR6 (mCXCR6)-transfected cells with

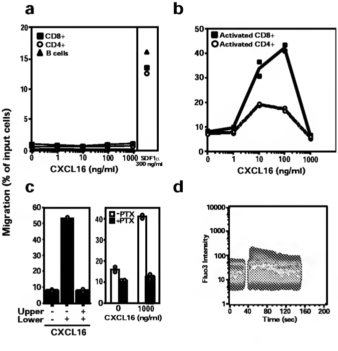

Figure 6. Migration of activated T cells to CXCL16. Results of migration assay are presented as the percentage of input cells of each cell type migrating to the lower chamber of a transwell filter. **(a)** Lack of detectable migration of T and B cells to recombinant CXCL16-Flag containing the chemokine domain. SDF1α was used as a positive control. **(b)** Response of *in vitro* activated CD8 and CD4 T cells to recombinant CXCL16. **(c) Left**, failure of activated T cells to migrate in the absence of CXCL16 gradient. CXCL16 was added to the upper or lower chamber of the transwell as indicated. **Right**, inhibition of CXCL16-induced migration by pretreatment of activated T cells with pertussis toxin (PTX). **(d)** CXCL16-induced calcium flux in Fluo3-loaded activated CD8 T cells measured by flow cytometry. The increase in fluorescence intensity of the intracellular indicator in the population of cells after addition of soluble CXCL16 (at 40 s) to 1 μg/ml is shown as a density plot.

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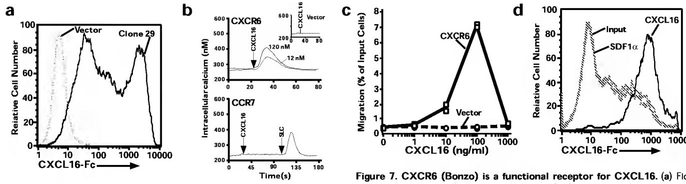

Figure 7. CXCR6 (Bonzo) is a functional receptor for CXCL16. (**a**) Flow cytometric analysis showing specific binding of CXCL16-Fc to HEK293 cells transiently transfected with a representative cDNA clone (clone 29) obtained from expression cloning, but not to cells transfected with empty vector. (**b**) CXCL16 induces a dose-dependent calcium flux in Indo-1–loaded, CXCR6-transfected HEK293cells, but not in cells transfected with CCR7 or empty vector. As a positive control for CCR7-transfected cells, calcium flux induced by its ligand, SLC, is shown. (**c**) CXCL16 elicits a strong chemotactic response in CXCR6-transfected Jurkat cells when compared with cells transfected with empty vector. (**d**) CXCR6 expression on cells before migration (input) and after chemotaxis to 0.1 μg/ml of CXCL16 (CXCL16) or 0.3 μg/ml SDF1α (SDF1α).

several other murine chemokines did not result in mobilization of intracellular calcium, in agreement with reports for the human receptor[14–16]. In chemotaxis assays, Jurkat cells transfected with CXCR6 showed a strong response to CXCL16 (**Fig. 7c**). Analysis of CXCR6 expression on the transfected cells before and after migration showed that only cells that highly expressed this receptor migrated to CXCL16 (**Fig. 7d**). The chemotaxis of activated, but not naïve, CD8 cells to CXCL16 (**Fig. 6**) was consistent with a requirement for high receptor levels to migrate to this chemokine.

Human Bonzo is expressed weakly on unstimulated CD8 T cells and more strongly on activated CD8 and CD4 T cells and in T cell lines[14–16,21], in agreement with the pattern of mCXCL16-Fc staining of mouse cells (**Fig. 5**). Therefore, our studies are consistent with Bonzo (CXCR6) functioning in mice and humans as the principal CXCL16 receptor.

Discussion

In the above studies, we identified a chemokine, CXCL16, which represents the only CXC chemokine to date that exists in a transmembrane form. CXCL16 was expressed on T cell zone DCs and sinus-associated cells of the splenic red pulp. Because the T cell–expressed HIV coreceptor, Bonzo, acts as a CXCL16 receptor, Bonzo was renamed CXCR6. These findings indicate that CXCL16 and CXCR6 function in DC–T cell interactions and in regulating T cell migration in the splenic red pulp. CXCL16 was also expressed in the thymic medulla and in several nonlymphoid tissues, indicating roles in thymocyte development and effector T cell trafficking.

The function of DCs as antigen presenting cells requires that they interact efficiently with naïve and activated T cells. Expression of T cell–attracting chemokines is likely to be one mechanism contributing *in vivo* to the efficiency of DC–T cell encounter; DCs make several T cell–attracting chemokines[22–26]. We show in this study that CD11c$^+$ DCs isolated from spleen and lymph nodes constitutively express CXCL16. By immunohistochemistry, we also found that CXCL16$^+$ cells, which most likely correspond to DCs, are detectable in T cell areas of spleen and lymph nodes. The presence of CXCL16 on both T zone DCs and thymic medullary cells indicates that this molecule may play a role in strengthening T cell–antigen presenting cell interactions at critical points in initiation of immune responses and during thymic selection of the T cell repertoire.

Several studies have shown that CD4 T cells can enhance activation of CD8 T cells by "licensing" DCs to become competent antigen presenting cells for CD8 T cells[27]. Inflammatory mediators such as LPS, poly(I)•poly(C) and stimulating antibodies to CD40 can substitute for the licensing action of CD4 T cells on DCs[27]. Although all of these mediators up-regulate costimulatory molecule CD86 (B7.2) on DCs, this molecule is necessary, but not sufficient, to account for enhanced activation of CD8 T cells by licensed DCs[28]. In this study we show that inflammatory stimuli that are known to license DCs for CD8 cell activation are effective in up-regulating CXCL16. We also demonstrate that naïve CD8 T cells constitutively express CXCL16 receptors and that receptor expression is strongly up-regulated on CD8 T cells during activation. These features indicate that CXCL16 may contribute to the ability of DCs to function as effective stimulators of CD8 T cells.

A striking and unusual property of CXCL16 is its distinct distribution in splenic red pulp. Although most splenic lymphocytes reside in the white pulp, notable numbers of T and B cells are found distributed in the red pulp[9]. Naïve CD8 cells are present in the red pulp at greater frequency than CD4 cells[10] and activated CD8 cells accumulate in the splenic red pulp during and following viral infections[11,29]. Although a few chemokines, including IP10 and MIG, have been detected in red pulp macrophages during inflammatory states, these molecules are only transiently expressed[30,31]. We show that CXCL16 is constitutively expressed in the red pulp and that it is up-regulated by inflammatory mediators. CXCL16 is expressed on contiguous elongated cells that seem to form distinct linear and often branching channels leading to red pulp sinuses. Because the CXCL16-expressing cells resemble sinusoidal endothelial cells[32,33], this pattern of expression places CXCL16 in a position that may allow regulation of T cell traffic through the red pulp sinusoids. By immunohistochemistry, red pulp CD8 cells were often seen in close contact with CXCL16-expressing cells, supporting the notion that CXCL16 and CXCR6 contribute to the trafficking of CD8 cells in this compartment. CXCL16 might function to retain T cells in the red pulp by preventing their exit into sinusoids or, alternatively, the chemokine might help guide the exit of T cells from the spleen.

The high expression of CXCL16 receptors by IELs and the presence of CXCL16 mRNA in small intestine indicates that CXCL16 and CXCR6 might have roles in retaining T cells in the intestinal epithelium. Previous analysis of human CXCR6 (Bonzo) showed detectable expression in gut[15], consistent with the notion that IELs express the receptor. IELs also express CX3CR1, and CX3CL1 has

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been identified on epithelial cells[34]. Therefore, the two transmembrane chemokines may function together to recruit and retain T cells in the epithelium. NKT cells also express high amounts of the CXCL16 receptor. NKT cells are found in spleen and represent a notable fraction of the T cells in the liver[35]. Future studies will need to investigate whether splenic or hepatic CXCL16 has a role in NKT cell retention or activation. The expression of CXCL16 in peripheral tissues may also have a role in recruitment or adhesion of activated CD8 and CD4 T cells. Preliminary results show that, as in spleen, CXCL16 is increased in liver following injection of poly(I)•poly(C) or anti-CD40 (M. Matloubian and J. G. Cyster, unpublished data), providing support for the notion that CXCL16 functions in peripheral tissues during inflammation.

CXCR6 was first characterized as a coreceptor for cellular entry of SIV and HIV[15,16,36]. Subsequent analysis has shown that although this coreceptor is able to support efficient replicative infection by SIV, it is weak at supporting replicative infection by most HIV isolates, despite often allowing viral binding and entry[37,38]. Therefore, it is presently unclear whether CXCR6 expression by IELs and activated T cells is likely to contribute positively to viral infection. An interesting consideration is that by supporting viral binding and entry, but not allowing efficient replicative infection, high expression of CXCR6 might reduce the sensitivity of T cells expressing CCR5 or CXCR4 to productive infection by HIV.

Together with CX3CL1, CXCL16 is the second member of the chemokine family with a transmembrane domain. Although the significance of transmembrane expression of chemokines is not yet clear, it is interesting that several other families of guidance factors, including the semaphorins and the ephrins, contain both membrane-tethered and secreted members[39,40]. Most secreted guidance factors, including chemokines, interact strongly with proteoglycans in the extracellular microenvironment. It will be interesting in future studies to understand how transmembrane expression, *versus* tethering through proteoglycans, influences the range of *in vivo* activities of chemokine molecules.

Methods

Clone identification, chromosomal localization, sequence and northern blot analyses. The LEADS platform for clustering and assembly of ESTs (http://www.labonweb.com/site-html/leads_overview_toc.html) was used to analyze Genbank version 106.0 and led to identification of a putative transcript encoding the human CXCL16. The following ESTs contain portions of human CXCL16 sequence: AA290712, AA130776, AA416552, AA149359, AA577696. Primers were designed from the assembled sequence and used in PCR to obtain the hCXCL16 sequence from a spleen cDNA library. The sequence of the 2750-bp long PCR product was identical to the predicted sequence. Using the human CXCL16 sequence as a BLAST query, we searched the National Center for Biotechnology Information (NCBI) EST database, which led to the identification of IMAGE consortium clones 1364724 and 1363378 as those encoding the mouse CXCL16. Both clones were obtained from Genome Systems, Inc. (St. Louis, MO) and sequenced. A profile search using the PROSITE database resulted in a match to profile PS50295 (small cytokines) in residues 24–119 of hCXCL16 and residues 1–97 of mCXCL16. Using PCR with several sets of specific primers on genomic DNA from the G3 radiation hybrids of Stanford, hCXCL16 was mapped to chromosome 17, between sequence-tagged sites D17S816E and D17S1854. A BLAST search performed against the genomic database also localized the sequence to chromosome 17 (GenBank AC027820). Northern blot analysis was done as described[22] using a 1-kb fragment of EST 1364724 encoding the full length mCXCL16, or mouse elongation factor (EF)-1α, as probe.

Biochemical analysis of CXCL16. To construct a full-length, amino-terminal Flag-tagged CXCL16, the region of mouse CXCL16 cDNA encoding amino acids 26–246 was cloned with PCR primers that introduced a 5′ *Sal*I site and a 3′ *Hind*III site. This fragment was ligated in-frame to a *Bam*HI/*Sal*I fragment encoding the prolactin signal peptide, followed by a Flag epitope[22]. The resulting DNA encoding prol/Flag/CXCL16 was inserted into a CMV promoter-driven expression vector, pRK5[41]. HEK293 cells were transfected with either pRK5-prol/Flag/CXCL16 or with the empty vector using LipofectAMINE PLUS reagent (Gibco, Grand Island, NY). Supernatants were collected 36–48 h post-transfection and cells were lysed in 1% NP40, 120 mM sodium chloride, 50 mM Tris pH 8.0, 1 mM benzamidine, 1 mM EDTA, 6 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin and 10 μg/ml of apoprotin. Flag-tagged proteins were immunoprecipitated with anti-Flag agarose (Sigma), resolved by 12.5% SDS-PAGE, transferred to PVDF membrane (Millipore, Bedford, MA) and probed with either biotin-coupled anti-Flag (Sigma) followed by strepavidin-horseradish peroxidase (Amersham), or with affinity-purified rabbit antiserum to CXCL16 followed by donkey anti-rabbit horseradish peroxidase (Amersham). Visualization was with enhanced chemiluminescence (ECL) or ECL+substrate (Amersham). For treatment with O-sialoglycoprotein endopeptidase, cells were washed and resuspended in RPMI with 0.1% bovine serum albumin at 10^7 cells/ml and incubated with and without 120 μg *Mannheimia haemolytica* O-sialoglycoprotein endopeptidase (Cedarlane, Ontario, Canada) at 37 °C for 1 h. Flag-tagged proteins were immunoprecipitated from the supernatants with anti-Flag agarose. Tissue lysates were prepared by homogenizing whole tissues in the above lysis buffer and were cleared of nuclear debris by centrifugation at 30,000*g* for 10 min.

Production of soluble CXCL16. The signal peptide and chemokine domain of mouse CXCL16 (aa1–114) were cloned using a 5′ PCR primer containing a *Bam*HI site and a 3′ oligonucleotide primer containing a *Hind*III site as well as an in-frame carboxy-terminal 8-amino acid Flag sequence, DYKDDDDK, and inserted into pRK5. HEK293 cells were transfected with this Flag-CXCL16 construct using LipofectAMINE PLUS (Gibco). Presence of a Flag-containing protein of the expected ~10-kD molecular mass in supernatants of transfected cells was confirmed by western blot. The recombinant chemokine was affinity-purified from culture supernatants with an agarose-coupled anti-Flag column (Sigma).

Production of CXCL16-Fc fusion protein. A PCR fragment encoding amino acids 1–122 of mCXCL16, generated using a 5′ primer containing a *Sac*I site and a 3′ primer containing a splice donor sequence and a *Hind*III site, was inserted into a vector (gift of P. Lane, Birmingham, UK) containing human IgG1 hinge, CH2 and CH3 domains as well as selection marker XGPRT[42]. This CXCL16-Fc construct was electroporated into mouse plasmacytoma cell line J558L and cells were selected for mycophenolic acid resistance[42]. Western blot analysis using either anti-human or anti-CXCL16 rabbit antiserum identified the same protein in culture supernatants, confirming the secretion of CXCL16-Fc fusion protein. This protein was further affinity-purified using a protein A-Sepharose column. For cell surface staining with CXCL16 fusion protein, spleen cells were first incubated with unconjugated anti-CD16/CD32 (Fc block) and then stained with the fusion protein. Cells were then sequentially stained with PE-conjugated goat anti-human–Fcγ (Jackson ImmunoResearch, West Grove, PA) that had been absorbed to 4% mouse and rat serum, then with other antibodies as indicated.

Production of rabbit antiserum to mCXCL16. A PCR fragment of mCXCL16 encoding amino acids 26–159 was cloned into the *Nde*I and *Xho*I sites of pET-23b (Novagen, Madison, WI) in-frame with a C-terminal His-tag sequence and expressed in *Escherichia coli*. The His-tagged protein was purified by binding to a NiNTA agarose column (Qiagen, Valencia, CA) according to manufacturer's instructions, then eluted with 200 mM imidazole. The purified material was resolved on continuous 15% SDS-PAGE and a dominant band of ~18 kD corresponding to the expected size of the cloned protein was excised and used in immunization of rabbits. Immunization and bleeding of rabbits was carried out at AnimalPharm Services, Inc. (Healdsburg, CA). Each rabbit underwent two cycles of immunization with 150 μg purified protein in complete Freund's adjuvant followed by five weekly boosts with 100 μg protein in incomplete Freund's adjuvant. The serum from the final bleed was affinity-purified using CXCL16-Fc coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). When used to probe western blots, the affinity-purified antiserum showed strong reactivity to recombinant CXCL16 but did not recognize other murine chemokines, ELC, SLC or BLC.

Immunohistochemistry and cell surface staining. Frozen sections (7 μm) were fixed for 10 min in 4 °C acetone, dried overnight, then stained, developed and photographed as described[22]. Staining with the affinity-purified rabbit anti-CXCL16 was detected using biotinylated goat anti-rabbit (PharMingen) that was absorbed to 1% mouse and rat serum. In control experiments in which the antiserum was preincubated with CXCL16-Fc for 4 h at 4 °C and then subjected to high-speed centrifugation to remove immune complexes, staining was reduced to background levels. Flow cytometry was done on a Becton Dickinson FACScan. Spleen single cell suspensions enriched in DCs were prepared as described[24]. For some experiments, mice were injected intraperitoneally with 300 μl of one of the following agents: PBS, LPS (50 μg), poly(I)•poly(C) (200 μg) (Sigma) or anti-CD40 clone FGK (100 μg).

Chemotaxis. Chemotaxis assays were done with 5-μm transwells (Corning Costar Corp., Corning, NY) as described[22]. In some experiments, cells were incubated with pertussis toxin (List Biological Labs, Campbell, CA) at 200 ng/ml for 1 h at 37 °C, then washed and used in the assay. For *in vitro* activation of T cells, spleen and lymph node single cell suspensions were incubated at 10^6 cells/ml in anti-CD3 (2C11) and anti-CD28 (clone 37.51, PharMingen)–coated plates in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, streptomycin (50 μg/ml), 10 mM HEPES, 50 μM 2-mercaptoethanol and mouse IL-2 (4 ng/ml) (PharMingen) for 5–6 days. The activated T cells were then rested in the above medium supplemented with 1–2 ng/ml of IL-2 for 4–6 days.

EXHIBIT D

Expression cloning of CXCR6. Mouse IL-2–activated T cell (LAK) cDNA library was produced using standard methods. Briefly, mRNA was purified from C57BL/6 mouse LAK cells using Fast Track mRNA isolation kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized in the presence of methyl-dCTP from mRNA using a poly(dT) primer containing a cryptic 3′ *Xho*I site and the Stratagene (La Jolla, CA) ZAP-Express cDNA library synthesis kit. After blunt-end ligation of precut *Eco*RI adaptors, the methyl-dCTP–cDNA was digested with *Xho*I. Digested cDNA was separated from unligated adaptors and short oligonucleotide cleavage products using Sephadex G25 chromatography. The purified, adapted cDNA was directionally ligated into *Eco*RI and *Xho*I sites of the episomal expression vector pMET7, transformed into DH10B ElectroMAX *E. coli* (Gibco), amplified on LB-ampicillin plates and collected. Library DNA was purified using Qiagen-tip 500 columns. HEK293 cells were transiently transfected with this library using the LipofectAMINE reagent (Gibco). Cells were released from tissue culture flask with PBS containing 0.5 mM EDTA 36–48 h after transfection. Any expressed Fc receptor was blocked by staining cells with FITC-labeled anti-CD16/CD32 (PharMingen). Cells were then sequentially stained with CXCL16-Fc and PE-labeled goat anti-human–Fcγ. Cells were sorted using a Becton-Dickinson FACSVantage SE and the top 0.1% of cells positive for CXCL16-Fc staining and negative for Fc receptor were collected. Plasmid DNA was extracted from these cells, electroporated into DH10B ElectroMAX *E. coli* and plated on ampicillin-containing LB plates. Plasmid DNA was extracted from the transformed bacteria using Qiagen-tip 500. HEK293 cells were transfected with this DNA and the cycle of selection by cell sorting was repeated for a total of four times. After the last round, pools of clones, then individual clones, were screened for binding to CXCL16-Fc. Several positive clones were sequenced and the nucleotide sequence of murine CXCR6 obtained in this manner has been deposited at GenBank (accession number AF301018).

Calcium flux. HEK293 cells were loaded with Indo-1 AM (Molecular Probes, Eugene, OR) and calcium mobilization was measured in a fluorimeter as described[22]. Activated T cells were loaded with 1 μg/ml of Fluo3 (Molecular Probes), stained with PE-conjugated anti-CD8 and fluorescence measurements were obtained using a FACScan (Becton Dickinson).

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1. Oppenheim, J. J., Zachariae, C. O. C., Mukaida, J. & Matsushima, K. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu. Rev. Immunol.* **9,** 617–648 (1991).
2. Cyster, J. G. Chemokines and cell migration in secondary lymphoid organs. *Science* **286,** 2098–2102 (1999).
3. Zlotnik, A., Morales, J. & Hedrick, J. A. Recent advances in chemokines and chemokine receptors. *Crit. Rev. Immunol.* **19,** 1–47 (1999).
4. Zlotnik, A. & Yoshie, O. Chemokines: a new classification system and their role in immunity. *Immunity* **12,** 121–127 (2000).
5. Pan, Y. *et al.* Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation *Nature* **387,** 611–617 (1997). (Erratum: *Nature* **389,** 100 (1997).)
6. Bazan, J. F. *et al.* A new class of membrane-bound chemokine with a CX3C motif. *Nature* **385,** 640–644 (1997).
7. Baggiolini, M., Dewald, B. & Moser, B. Human chemokines: an update. *Annu. Rev. Immunol.* **15,** 675–705 (1997).
8. Unutmaz, D., KewalRamani, V. N. & Littman, D. R. G protein-coupled receptors in HIV and SIV entry: new perspectives on lentivirus-host interactions and on the utility of animal models. *Semin. Immunol.* **10,** 225–236 (1998).
9. Pellas, T. C. & Weiss, L. Migration pathways of recirculating murine B cells and CD4+ and CD8+ T lymphocytes. *Am. J. Anat.* **187,** 355–373 (1990).
10. Willführ, K. U. & Westermann, J. Absolute numbers of lymphocyte subsets migrating through the compartments of the normal and transplanted rat spleen. *Eur. J. Immunol.* **20,** 903–911 (1990).
11. Potsch, C., Vohringer, D. & Pircher, H. Distinct migration patterns of naive and effector CD8 T cells in the spleen: correlation with CCR7 receptor expression and chemokine reactivity. *Eur. J. Immunol.* **29,** 3562–3570 (1999).
12. Trinchieri, G. Biology of natural killer cells. *Adv. Immunol.* **47,** 187–376 (1989).
13. Smith, K. G. C., Hewitson, T. D., Nossal, G. J. V. & Tarlinton, D. M. The phenotype and fate of the antibody-forming cells of the splenic foci. *Eur. J. Immunol.* **26,** 444–448 (1996).
14. Liao, F. *et al.* STRL33, a novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1. *J. Exp. Med.* **185,** 2015–2023 (1997).
15. Deng, H. K., Unutmaz, D., KewalRamani, V. N. & Littman, D. R. Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature* **388,** 296–300 (1997).
16. Loetscher, M. *et al.* TYMSTR, a putative chemokine receptor selectively expressed in activated T cells, exhibits HIV-1 coreceptor function. *Curr. Biol.* **7,** 652–660 (1997).
17. Hofmann, K., Bucher, P., Falquet, L. & Bairoch, A. The PROSITE database, its status in 1999. *Nucleic Acids Res.* **27,** 215–219 (1999).
18. Cyster, J. G., Shotton, D. M. & Williams, A. F. The dimensions of the T lymphocyte glycoprotein leukosialin and identification of linear protein epitopes that can be modified by glycosylation. *EMBO J.* **10,** 893–902 (1991).
19. Abdullah, K. M., Udoh, E. A., Shewen, P. E. & Mellors, A. A neutral glycoprotease of Pasteurella haemolytica A1 specifically cleaves O-sialoglycoproteins. *Infect. Immun.* **60,** 56–62 (1992).
20. Hooper, N. M., Karran, E. H. & Turner, A. J. Membrane protein secretases. *Biochem. J.* **321,** 265–279 (1997).
21. Edinger, A. L. *et al.* Use of GPR1, GPR15, and STRL33 as coreceptors by diverse human immunodeficiency virus type 1 and simian immunodeficiency virus envelope proteins. *Virology* **249,** 367–378 (1998).
22. Ngo, V. N., Tang, H. L. & Cyster, J. G. Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J. Exp. Med.* **188,** 181–191 (1998).
23. Sallusto, F. *et al.* Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* **29,** 1617–1625 (1999).
24. Tang, H. L. & Cyster, J. G. Chemokine upregulation and activated T cell attraction by maturing dendritic cells. *Science* **284,** 819–822 (1999).
25. Papadopoulos, E. J. *et al.* Fractalkine, a CX3C chemokine, is expressed by dendritic cells and is up-regulated upon dendritic cell maturation. *Eur. J. Immunol.* **29,** 2551–2559 (1999).
26. Kanazawa, N. *et al.* Fractalkine and macrophage-derived chemokine: T cell-attracting chemokines expressed in T cell area dendritic cells. *Eur. J. Immunol.* **29,** 1925–1932 (1999).
27. Lanzavecchia, A. License to kill. *Nature* **393,** 413–414 (1998).
28. Ridge, J. P., Di Rosa, F. & Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* **393,** 474–478 (1998).
29. Haanen, J. B. *et al.* Systemic T cell expansion during localized viral infection. *Eur. J. Immunol.* **29,** 1168–1174 (1999).
30. Ohmori, Y. *et al.* Tumor necrosis factor-alpha induces cell type and tissue-specific expression of chemoattractant cytokines *in vivo*. *Am. J. Pathol.* **142,** 861–870 (1993).
31. Amichay, D. *et al.* Genes for chemokines MuMig and Crg-2 are induced in protozoan and viral infections in response to IFN-γ with patterns of tissue expression that suggest nonredundant roles *in vivo*. *J. Immunol.* **157,** 4511–4520 (1996).
32. Bordessoule, D., Gaulard, P. & Mason, D. Y. Preferential localisation of human lymphocytes bearing γδ T cell receptors to the red pulp of the spleen. *J. Clin. Pathol.* **43,** 461–464 (1990).
33. Balazs, M., Grama, L. & Balogh, P. Detection of phenotypic heterogeneity within the murine splenic vasculature using rat monoclonal antibodies IBL-7/1 and IBL-7/22. *Hybridoma* **18,** 177–182 (1999).
34. Muehlhoefer, A. *et al.* Fractalkine is an epithelial and endothelial cell-derived chemoattractant for intraepithelial lymphocytes in the small intestinal mucosa. *J. Immunol.* **164,** 3368–3376 (2000).
35. Bendelac, A., Rivera, M. N., Park, S. H. & Roark, J. H. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* **15,** 535–562 (1997).
36. Alkhatib, G., Liao, F., Berger, E. A., Farber, J. M. & Peden, K. W. A new SIV co-receptor, STRL33 (letter; see comments). *Nature* **388,** 238 (1997).
37. Pohlmann, S., Krumbiegel, M. & Kirchhoff, F. Coreceptor usage of BOB/GPR15 and Bonzo/STRL33 by primary isolates of human immunodeficiency virus type 1. *J. Gen. Virol.* **80,** 1241–1251 (1999).
38. Zhang, Y. J. & Moore, J. P. Will multiple coreceptors need to be targeted by inhibitors of human immunodeficiency virus type 1 entry? *J. Virol.* **73,** 3443–3448 (1999).
39. Van Vactor, D. V. & Lorenz, L. J. Neural development: the semantics of axon guidance. *Curr. Biol.* **9,** R201–204 (1999).
40. Holder, N. & Klein, R. Eph receptors and ephrins: effectors of morphogenesis. *Development* **126,** 2033–2044 (1999).
41. Reif, K., Nobes, C. D., Thomas, G., Hall, A. & Cantrell, D. A. Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. *Curr. Biol.* **6,** 1445–1455 (1996).
42. Lane, P. *et al.* Activated human T cells express a ligand for the human B cell-associated antigen CD40 which participates in T cell-dependent activation of B lymphocytes. *Eur. J. Immunol.* **22,** 2573–2578 (1992).